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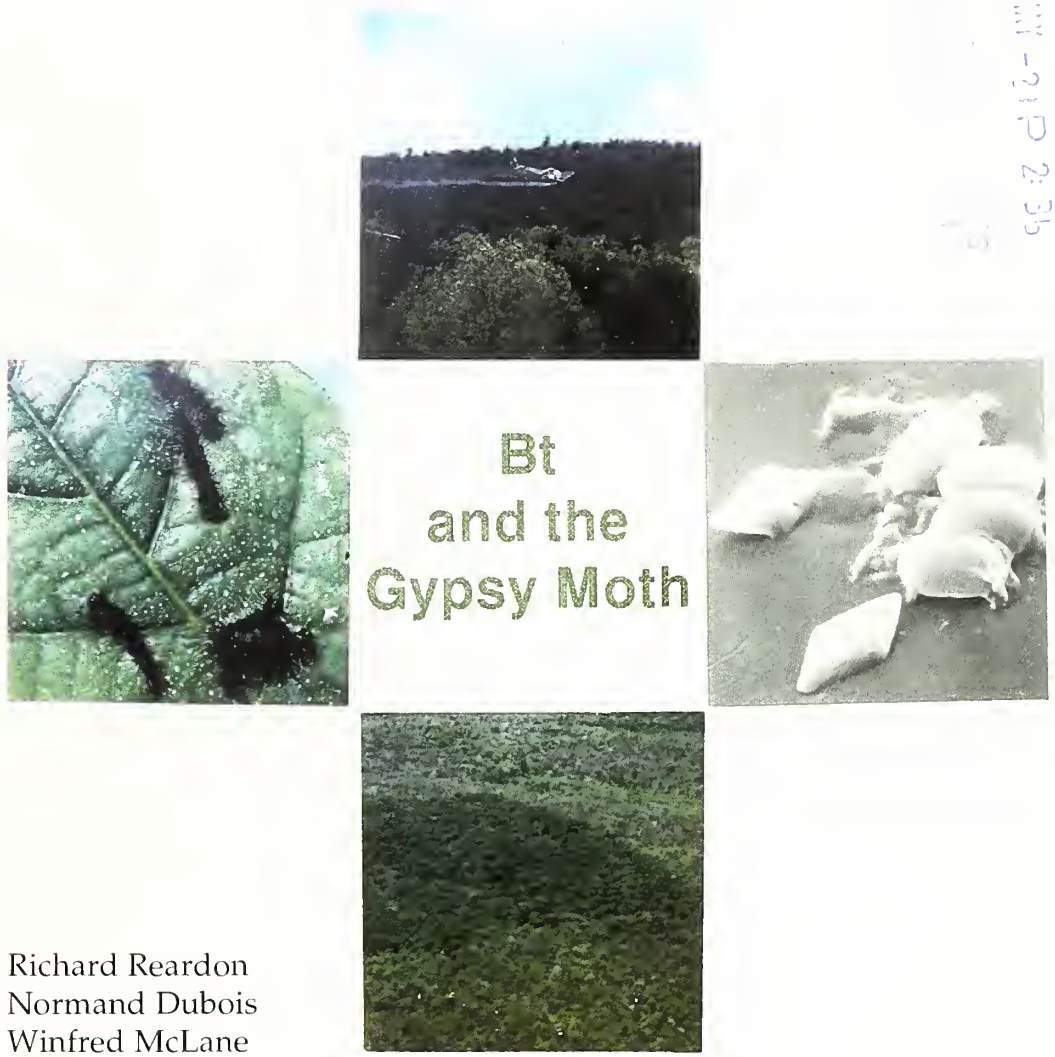
Bacillus thuringiensis

Bacillus thuringiensis for Managing Gypsy Moth: A Review



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We dedicate this publication to William G. Yendol (1931-1993), whose research contributed to the development of *Bacillus thuringiensis* in forestry. We miss his friendship and advice.

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Preface

This publication is the first in a series (FHM-NC-01-94) supported by the USDA Forest Service National Center of Forest Health Management in Morgantown, West Virginia.

The National Center of Forest Health Managment was established in April 1993 for the purpose of aeeelerating development and use of environmentally aeeeptable teehnologies to improve the health of Ameriea's forests. The Center has national responsibility to address teehnology needs that are a key for sueeessful integrated pest management of major forest inseets and diseases, and for the sueeessful management of forest eeosystems.

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Bacillus thuringiensis for Managing Gypsy Moth: A Review

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Introduction

The *Bacillus thuringiensis* Berliner group of bacteria (commonly referred to as *Bt*) is receiving increasing attention for use in integrated pest management programs for agricultural and forest insect pests and insect vectors of human and other mammalian transmissible diseases. Taxonomically, these entomopathogenic (causing disease in insects) bacteria are in the family Bacillaceae and are members of the genus *Bacillus*. Typically, they are rod-shaped, form a spore and are motile by flagellae (whip-like appendages). In addition and unique to this species, they form a protein crystal next to the spore at the time of sporulation. *B. thuringiensis* occurs naturally in numerous species of agricultural and forest insects and is a component of the soil microbiota worldwide (Martin and Travers 1989). Many different strains of *Bt* have been isolated from soils, however, most strains used in commercial production of microbial insecticides have been isolated from diseased insects (DeLucca et al. 1981).

Bacillus thuringiensis was first isolated from diseased silkworm, *Bombyx mori* (L.), larvae in Japan in 1901 by Ishiwata who named it *Bacillus sotto*. In 1911, a German entomologist named Berliner isolated another variety of this bacterium from diseased Mediterranean flour moths, Ephestia *(=Anagasta) kuehniella* (Zeller), that were found in stored grain in Thuringia. In 1915, he named it *Bacillus thuringiensis* Berliner, recorded the first scientific description of the bacterium (Ishiwata did not formally describe the organism he found), and is credited with naming it (Beegle and Yamamoto, 1992). This culture (Berliner strain of variety *thuringiensis*) was lost, and in 1927 Mattes reisolated the same organism from the same host as did Berliner. Mattes' isolate was widely distributed in most of the early commercial *Bt*-based products and to date, it is the representative strain for the type species of these crystal-forming bacteria.

Through the research and promotional efforts of E. A. Steinhaus in the early 1950's, development of *B. thuringiensis* var. *thuringiensis* proceeded quickly and led to commercial production and extensive research. Kurstak, in 1962, isolated another variety of *B. thuringiensis* that was effective primarily against Lepidoptera and named it *kurstaki*. In 1970, Dulmage isolated another more potent strain of this variety from diseased mass-reared pink bollworm, *Pectinophora gossypiella* (Saunders), larvae and coded it the HD-1 strain (Dulmage 1970). This strain, often referred to by its acronym "*Btk*", became commercially available through Abbott Laboratories as Dipel in the early 1970's. Since this strain is active and more potent than previous strains against numerous lepidopteran species, it is used today for production of most formulations of *Bt* that are used to

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control defoliating Lepidoptera in North America. What began in the 1950's as a collection of less than a dozen *Bt* strains, now has grown to over a 1,000 strains of different varieties maintained at the *Bacillus thuringiensis* Culture Repository at the Northern Regional Research Laboratory (N.R.R.L) of the United States Department of Agriculture (USDA), Agricultural Research Service (ARS), at Peoria, IL. In addition, a large number of cultures and variants developed through genetic manipulations are held by industry.

First attempts to use *B. thuringiensis* for insect control took place in the late 1920's against the gypsy moth, *Lymantria dispar* (L.), in the northeastern United States (Metalnikov and Chorine 1929) and in the early 1930's against the European corn borer, *Ostrinia nubilalis* (Huber), in eastern Europe. The first commercial *B. thuringiensis* product, Sporeine, was available in 1938 in France (Entwistle et al. 1993). In the United States, the first commercial *B. thuringiensis* product, Thuricide (Pacific Yeast Products=Bioferm Corp.), became available for testing in 1958. In 1960, the United States Food and Drug Administration (FDA) granted a full exemption from residue tolerances, and the first formulated *Bt* product was registered in 1961, under the trade name of Thuricide by the Pesticide Regulation Division of the USDA.

Since 1980, approximately 1.7 million hectares (ha) (4.2 million acres) have been treated with *B. thuringiensis* var. *kurstaki (Bt)* in the eastern United States as part of the Federal, State, and County Gypsy Moth Cooperative Suppression Program. During this interval, the use of *Bt* against the North American gypsy moth (European strain that was introduced and established in the United States since the 1860's) ranged from a low of 6.4 percent of the total area treated with *Bt* to a high of 79.5 percent in any single year (Machesky 1993). A wide range of aircraft types and spray equipment were used to apply various doses, rates and formulations of *Bt*. Generally one application was used in normal spray operations. However, two or three applications were commonly used in eradication efforts in Oregon (1985-87) and Utah (1988-1993) against the North American gypsy moth and, in Washington and Oregon (1992), on approximately 200,000 ha against the Asian strain of gypsy moth. The Asian gypsy moth was introduced around 1990 via cargo shipping activities from the Siberian coast of Russia through British Columbia. Also, multiple applications (two to three) of *Bt* are planned (1994) to eradicate an infestation of European, Asian and hybrid strains of the gypsy moth (introduced via military cargo shipped from Germany) on approximately 50,000 ha in eastern North Carolina. In Ontario, Canada, between 1985 and 1990, 204,000 ha were treated with *Bt* to control North American gypsy moth.

The worldwide market for *Bt* var. *kurstaki*-based products for forestry and agriculture is estimated to be about US$60 million-80 million per year. At present, the single largest market for these products is against forest insect pests in North America (Beegle and Yamamoto 1992).

This publication describes the biology and mode of action of *Bt*; field uses against gypsy moth including application, efficacy, safety, effects on nontargets, resistance, interaction with natural enemies; and new developments.

Biology

B. thuringiensis is commercially produced by liquid fermentation. Large vats (5,000- to 140,000-liter capacity) filled with water, proteins, carbohydrates (sugar) and other ingredients needed to sustain *Bt* growth, are seeded with a small amount of *Bt* and incubated under specific conditions. When cell replication is complete (i.e., 10 to 100 millionfold increase in 72-120 hours) (Figure 1A), a spore (or endospore, dormant stage of the bacteria), and a diamond-shaped protein inclusion referred to as the crystal (or parasporal body) are formed within the vegetative cell, which is now referred to as the sporangium (Figure 1B). At the completion of spore formation, the wall of the sporangium breaks down releasing both the spore and crystal (Figure 1C) into the surrounding growth medium (Dubois and Lewis 1981). The spores, crystals and other residual fermentation solids are then harvested, stabilized, standardized, and formulated into the commercial product. Therefore, commercial formulations of *Bt* contain both the spore and crystal as their entomopathogenic ingredients.

The crystal of *Bt* var. *kurstaki* is a bipyramidal protein matrix (Figure 1D) of large molecules of inactive protoxins. These are not toxic to insects until solubilized in the gut by the insect's digestive fluids and released as smaller proteins (delta-endotoxins), which are the true toxins. Therefore, the susceptibility of an insect to these toxins may in part, or perhaps entirely, depend on the insect's ability to solubilize and digest the crystal into its toxic subunits. In most lepidopteran pests, when ingested separately, the toxin subunits are the major cause of mortality, whereas the spore effect is minimal. However, some "pure spore" preparations can contain proteins on the spore coat that are homologous to the delta-endotoxins; these are toxic to some insects. During the vegetative growth phase of *Bt* in fermentation, some strains of *Bt* produce and release into the liquid fermentation medium, a water-soluble and heat-stable beta-exotoxin. Generally when ingested, this toxin, also known as *thuringiensin*, can have some toxic effects in birds as well as a broad spectrum effect on numerous insects and other invertebrates, particularly flies (it was once referred to as the fly knockdown factor). *Thuringiensin* apparently causes interference in ribonucleic acid (RNA) transcription (Johnson 1978). In North America, since commercial formulations of *Bt* are not allowed to contain beta-exotoxin at a level detectable with approved standard methods, each commercial batch is checked, and if the beta-exotoxin is present, it is rejected.

As part of its mode of action, *Bt* can germinate, multiply and resporulate in the infected insect's hemolymph (the blood of insects); however, vegetative cells, spores and crystals are not abundantly produced under such conditions. Since the insect integument (outer layer of an insect) does not rupture, spores and crystals are not released to contaminate foliage that might be consumed by other susceptible species. The dead insects usually fall to the ground, and the *Bt* toxins are degraded by normal environmental soil factors. Under favorable conditions, *Bt* spores can germinate and grow in moist soil, deriving essential nutrients from decaying plants. The spore can persist in soil (and other protected sites) longer than the crystal toxins (West et al. 1984) and typically can survive for several months and, under ideal conditions, for years. *Bt* can sporulate successfully to levels of more than 1 million spores per gram of soil (Saleh et al. 1969).

Unlike natural epizootics caused by nucleopolyhedrosis viruses (NPV's) and by the fungus, *Entomophaga maimaiga* (Hajek and Roberts 1992), natural epizootics caused by *Bt* have never been observed as a control factor for forest insect populations in nature. Consequently, to control insect populations, *Bt* must be applied annually in the manner of a conventional stomach-poison type of insecticide (Figure 2A). *Bt* cannot be expected to infect subsequent generations of the gypsy moth (Dubois et al. 1988).

Mode of Action

The mode of action of *Bt* is fairly complex and poorly understood. In susceptible insects, the alkaline midgut environment (pH>8.0) and proteolytic (protein-splitting) enzymes, dissolve ingested crystals and release smaller delta-endotoxins. These proteins, also known as the insecticidal crystal proteins (ICP's), recognize and bind to specific receptors on the cellular lining of the midgut. Depending on the *Bt* strain used, one or several different types of ICP's may be released from the crystal matrix. Once bound to the receptors, ICP's penetrate through the cell membrane and form ion-selective channels. The selective permeability characteristic of the cell membrane is then disrupted, causing the cell to absorb water, swell, and burst. This results in a perforation of the gut and leakage of gut content, including spores, into the hemolymph. At this point, gut paralysis (and in some cases, paralysis of the mouthparts) occurs, the larva stops feeding, and dies in a few hours to a few days (Figure 2B). In less susceptible species, the spore penetrates into the hemolymph where conditions of the hemolymph permit spore germination and bacterial (vegetative cell) multiplication to take place, resulting in a septicemia (a morbid condition), that contributes to or causes death. If a sublethal dose is ingested, the larva stops feeding, weight gain and development are stunted, and the larva suffers various nonlethal physiological effects. Regeneration of the damaged cells in the midgut can occur, and the larva may eventually recover and resume feeding (Fast and Regniére 1984).

Taxonomy

The 34 varieties (subspecies) of *Bt* are divided into 27 major antigenic groups (e.g., H1, H4, H14) plus 7 subgroups (e.g., H4a H4b) called serotypes (serovars). Of the 34 varieties, 22 are active against lepidopteran pests (de Barjac and Frachon 1990). Seventeen of the 22 varieties are insecticidal at 100 µg/ml of diet against gypsy moth (Dubois et al. 1989). The varieties of *Bt* are normally named for the insect or the area from which they were isolated or for some other item related to the isolate or its isolation. Serotype classification is based on flagellar (H) antigens of the vegetative cell. Another taxonomic scheme groups the *Bt* varieties into 14 serotypes based on the antigenic composition of the crystal (de Barjac and Bonnefoi 1962). Many of these serotypes exhibit different spectra of insecticidal activity (Dulmage et al. 1981, Dubois et al. 1989). In a recent review, Beegle and Yamamoto (1992) suggested that the taxonomic scheme based on only the flagellar antigens was insufficient. They pointed out that, in at least three cases, the scheme failed to separate distinct pathological or physiological types because they shared the same antigenic profile. These are the H4a4b (*Bt* varieties *sotto* and *dendrolimus*, H8a8b (*Bt* varieties *morrisoni* and *tenebrionis)* and H20 (*Bt* varieties *yunnanensis* and *pondicheriensis*). This then increases the classification to 37 varieties. Whether the pathological or biochemical profile should

be included in the taxonomic scheme is still unsettled, nonetheless the reader should be aware that especially *Bt* var. *tenebrionis* is usually referred to as a separate variety of *Bt*. With advancements in plasmid mapping, cloning and sequencing of toxin genes, and high-performance liquid chromatography, the taxonomic scheme of *Bt* probably will be revisited in the not too distant future.

Most *Bt* formulations produced before 1971 were prepared with *B. thuringiensis* var. *thuringiensis* (serotype 1). The HD-1 strain of *B. thuringiensis* var. *kurstaki*, which has been used extensively since 1971, is a serotype 3a3b, and the crystal has a fairly broad spectrum of activity against a large number of Lepidoptera. Other *Bt* varieties are commercially produced for use against specific groups of insects: (1) *Bt* var. *aizawai* (serotype 7) for use against other Lepidoptera, specifically the wax moth, *Galleria mellonella* (L.), and diamondback moth, *Plutella xylostella* (L.), (2) *Bt* var. *israelensis* (serotype 14) for use against Diptera, and (3) *Bt* var. *tenebrionis* and *morrisoni* (serotype 8a8b) for use against Coleoptera.

Potency of Formulations

Standardization

Initially, the potency (insecticidal activity) of *Bt* formulations was determined by spore counts. The number of spores in the preparation, however, is unrelated to its potency, and the variability associated with this method was extremely high. An international standard (designated E-61) for determining the potency of *Bt* preparations was devised at the Pasteur Institute and universally accepted in 1966. This standard, a dry powder preparation of *B. thuringiensis* var. *thuringiensis* (serotype 1) was defined to contain 1000 International Units of Insecticidal Activity (IU) per milligram of powder. With the development and use of the HD-1 strain, a second international standard, HD-1-S-1971, was developed using the HD-1 strain of *B. thuringiensis* var. *kurstaki* . It was accepted in 1972 as the standard for *Bt* products derived from that strain. Relative to the E-61 standard, HD-1-S-1971 was defined to contain 18,000 IUs per milligram of powder (*B. thuringiensis* var. *kurstaki* is 18 times more potent than *B. thuringiensis* var. *thuringiensis*). By late 1979, the supply of HD-1-S-1971 was depleted to a level that necessitated the development of a new HD-1 standard. The presently used *B. thuringiensis* var. *kurstaki* standard is HD-1-S-1980 and its potency was calculated at 16,000 IUs per milligram of powder (Beegle et al. 1986). A precise protocol must be followed when determining the potency of a *Bt* product. The potency of *Bt* preparations is determined by parallel bioassays of the product and the standard on artificial diet with 4-day-old cabbage looper, *Trichoplusia ni*, larvae (Beegle et al. 1986). Potency is the ratio of the LC_{50} of the standard to the test product, multiplied by the defined potency of the standard. The LC_{50} is the amount (i.e., lethal concentration) of material required to kill 50 percent of the insects exposed to it. The unit of measure used is either milligram or microgram and is calculated according to the equation shown below:

$$\frac{LC_{50}\ (\text{HD-1-S-1980})}{LC_{50}\ (\text{product})} \times 16{,}000\ \text{IU/mg} = \text{potency of the product in IU/mg}$$

The determined potency is used in quality control to ensure a product meets its labeled potency. Since insecticidal activity against diverse insect species varies greatly, this method often results in a misrepresentation of the actual efficacy against species other than the cabbage looper. Therefore, it is recommended that the label include also the quantity or percent concentration of the delta-endotoxin or the specific ICP and the recommended dose of formulated product per unit area for use against species for which it is registered.

Identity

Most labels of *Bt* products are fairly consistent and easy to read. Requirements established by the United States Environmental Protection Agency (EPA) make certain statements uniform and clear, such as the EPA Registration and Establishment Numbers, and the precautionary statements concerning disposal, cleanup, environmental and health effects. Where both protein toxin and spores may play a significant role in the expressed potency, however, the concentration of insecticidal content of a *Bt* product is not readily defined or quantified; and the chemical analysis of ICP quantification is not universal or consistent. Consequently, considerable discretion is exercised by industry as to the manner by which these requirements are written on the labels, and the registered and recommended doses for use against a particular pest. These discrepancies include the identity of the *Bt* used, its defined potency and toxin concentration, and the maximum allowed (registered) dose and use.

Identity —Formulations used against lepidopteran pests are almost all produced with *Bt* var. *kurstaki*, however, they may have different labels:

"*Bacillus thuringiensis* Berliner," simply identifies the genus *Bacillus thuringiensis* without specifying that it is *Bt* var. *kurstaki* (which it probably is).

"*Bacillus thuringiensis* var. (or subspecies) *kurstaki*" uses the terms "variety" and "subspecies" interchangeably.

"*Bacillus thuringiensis* strain XX-#" tells that the product is produced from a specific strain that was selected (probably after some genetic manipulation) for this product.

"*Bacillus thuringiensis* var. (subsp) *tenebrionis*" or "*israelensis*" would identify other *Bt* products such as those used against coleopteran or dipteran pests.

If the term "*Bacillus thuringiensis*" is not mentioned, then the product is probably another organism type (bacterium or plant) with some *Bt* gene(s) cloned into it.

Defined potency and toxin concentration — The bioassay with *Trichoplusia ni* normally defines the potency to a lepidopteran pest, and one may assume that the insecticidal activity is in *T. ni* units and its concentration is defined as International Units per milligram (mg) of powder. If the product is a liquid concentrate, there would be also some relationship to volume in terms of IU per gallon or liter. Some products may have the potency defined as

Spodoptera Units (SPU), *Leptinotarsa* Units (LTU) or International Toxin Units (ITU's). These units are used because these *Bt* varieties do not have any insecticidal activity against *T. ni*. They are usually used against insects such as armyworms, Colorado potato beetle or mosquitoes, respectively, and require these other insect species to measure insecticidal activity. The percentage of ingredient relative to total material is stated and may vary from 2 to 7 percent, the balance being inert ingredients. In addition, some labels may contain a measurement of toxin protein as percent protein, referring to the lepidopteran-active toxin(s) present in the crystal.

Maximum allowed (registered) dose and use — Generally,dose ranges are recommended in either weight or volume (sometimes in BIU) for crop types and the insect pest for which the product is registered. Also in this section, one may find a recommended dilution procedure. Every *Bt* product is registered for a maximum dose allowed. That dose may not be explicitly stated on the label; although, it may be the upper range of the recommended dose for use against a particular pest. Users who wish to use a product at higher than the recommended doses should consult the manufacturer to be assured that they are in compliance with EPA regulations and to prevent voiding the limited warranty that may appear on the label. Though not required, most labels contain other information (e.g., drop size, application timing) designed to facilitate or optimize the use of that product.

Laboratory Methods for Gypsy Moth

For potency determination, parallel bioassays with the international standard and the test preparation are usually conducted against one-day-old second-instar gypsy moth larvae (Dubois 1986). In an extensive study, Dubois et al. (1989) evaluated over 260 *Bt* strains against gypsy moth and spruce budworm. This study showed that proper comparison of formulations is best done against the international standard. The reason is primarily because differences in larval batches and variation in fermentation have a dramatic effect on the potency of a formulation.

Comparisons of preparations for efficacy need to take into consideration not only the LC_{50} but the slope of the regression as well. The slope shows the dose-response relationship over a range of doses, i.e., the regression coefficient. This information is important because many preparations have similar LC_{50} but differ significantly at the 95 percent level of effectiveness (that dose needed to kill at least 95 percent of the larvae, i.e., LC_{95}). It is at this level of effective insecticidal activity that is required for *Bt* to significantly reduce a pest population to acceptable levels.

Commercial Formulations

Commercial development of *Bt* formulations from 1961 through 1974 was summarized by Dubois and Lewis (1981). In the 1960's formulations were prepared primarily with *Bt* var. *thuringiensis*, as wettable powders, and sprayed as suspensions in oil (e.g., No. 2 fuel oil) or water. Also, they included a sticker (e.g., Chevron), a feeding stimulant (e.g., molasses) and a sunscreen. Application problems including short shelf- life, unstable suspension, nozzle clogging, uneven spray distribution; subsequently, poor potency were common with these formulations.

By the late 1960's suspendability and clogging problems were alleviated with the development of formulations that were flowable concentrates. At that time, the major thrust for improvement of field efficacy of *Bt* focused on isolating and developing new, more potent strains rather than improving formulation and application technology. In 1971, *Bt* var. *kurstaki* HD-1 strain became available for commercial development. During the early 1970's, using the HD-1 strain, Sandoz Inc. produced primarily aqueous based concentrates (e.g., Thuricide HPC) and Abbott Laboratories focused on refining readily suspendable wettable powder formulations. Adjuvants such as Nufilm-*Bt*, Santoquin, and Maywood were introduced into *Bt* formulations as stickers or sunscreens or both. Potency of the strain used, the potency of the formulation, and coverage and persistence on the foliage were all identified as critical components for successful use of *Bt* in forestry.

During the 1980's, formulation bio-characteristics (activity, stability, potency, concentration and dose-response relationship) were identified as significant factors affecting field efficacy. Although the recent formulations can stick to foliage after drying for 6-8 hours, a sticker additive is usually recommended when applying diluted tank mixes of *Bt* formulations because of the possibility of intense rainstorms. Bond, Plyac, and Rhoplex are the more commonly used stickers. NOT ALL FORMULATIONS ARE COMPATIBLE WITH ALL STICKERS, and users should consult the manufacturer when selecting a sticker. There is limited laboratory and field data on the compatibility of stickers as well as on how they affect efficacy of the different formulations (especially when applied undiluted) which limits their use in operational programs.

Four companies produce formulations of the HD-1 strain of *Bt* var. *kurstaki* for use against gypsy moth:

Company	Address/Contact	Formulation
Abbott Laboratories	North Chicago, IL 60064 Contact: 708-937-8813	Aqueous flowable suspensions -- Dipel 6AF and 8AF Nonaqueous emulsifiable suspensions -- Dipel 4L, 6L, 8Land 12L
Ecogen Inc.	Langhorne, PA 19047-1810 Contact: 215-757-1590	Oil flowable -- Condor OF (strain EG 2348)
Novo Nordisk Bioindustrials, Inc.	Danbury, CT 06813-1907 Contact: 800-283-3386	Aqueous flowable suspensions -- Foray 48B and Foray 76B
Sandoz Crop Protection	Des Plaines, IL 60018 Contact: 708-390-3820	Aqueous flowable suspensions -- Thuricide 32LV, 48LV and 64LV (also SAN-415 SC32LV, which is produced with the NRD-12 strain)

Field Use

Application

Numerous physical, biotic (e.g., population quality and density), climatic and physiographic (e.g., elevation) factors affect the efficacy of *Bt* (Harper 1974). The effective use of *Bt* to control gypsy moth involves their interaction of numerous factors. Fortunately, most of these factors can be selectively determined (e.g., population density, formulation) although a few are not accurately predicted (e.g., weather). A few of the more important factors are discussed in detail: mixing, application timing and technology, application dose and volume, and weather.

Mixing — Aqueous flowable formulations of *Bt* used against lepidopteran pests can be applied undiluted with most aerial spray equipment. Preparation of tank mixes with water or other diluents, other adjuvants, or dye markers require some precautionary measures. Dubois and Lewis (1981) recommend the following:

1. The pH range of the water used as the diluent should be between 5.5 - 7.5; a higher pH (i.e.,>pH8) could dissolve the crystal and release the unstable toxins, and a low pH (i.e., <pH5.0) could denature the proteins of the crystal. The pH of the water can be easily checked with pH-sensitive paper.
2. Chlorinated water should be avoided if possible (chlorination at drinking water levels is not a problem).
3. *Bt* should be tank-mixed immediately before use and not be prepared more than 72 hours before spraying (see Microbial Contaminants Section).
4. *Bt* should not be frozen or subjected to high temperatures (above 30°C) for any length of time.

Special consideration is needed when using nonaqueous formulations such as Dipel oil formulations. Before using these as undiluted tank mixes, all water must be removed from the ground handling and spraying systems. To avoid the formation of inverse emulsions, dilution of oil-based concentrates (i.e. Dipel L formulations) with water should always be done as follows: water (at least in a 50:50 ratio) should be added first into the mixing tank followed with the concentrate. After application, the following cleaning procedure is recommended (Fuseo 1993):

1. Remove in-line screens and nozzles or atomizers.
2. First, flush the entire pumping and aircraft spraying systems with diesel fuel.
3. Second, flush the system with water containing a detergent (e.g., Top Job Liquid Cleaner at 1qt/100 gal.).
4. Finally, rinse with water all in-line screens and nozzles, and replace.

Because of settling in containers during storage and shipment, all *Bt* formulations should be recirculated prior to use. When recirculating tank mixes, it is desirable to submerge the outlet hose under the liquid in the receiving tank to prevent excessive aeration.

When pumping from a tanker, the manhole of the tanker must always be open to prevent collapse of the tanker walls. If the tanker is being positioned for use over time, it must be placed on solid ground with the front support on heavy (4-6 inch thick) wooden boards and the outlet at the lowest point. If unloading from a compartmentalized tanker, always unload from the front compartment first and proceed towards the rear compartment. Reverse the procedure when loading into a compartmentalized tanker (Fusco 1993).

Timing — Timing of application is generally dictated by insect and foliage development (Dubois 1991). Three factors determine the timing of application: (1) the degree of foliage expansion, (2) larval stage of development, and (3) the size (biomass) of the larvae within an instar (there is an inverse relationship between susceptibility to *Bt* and larval size). Since *Bt* must be ingested to be effective (see Mode of Action section), target foliage should be sprayed, if possible, when its average expansion is at least 45 percent. Of greater importance is timing the application, when the majority of larvae have developed to late first or early second instar (early first instar larvae feed very little). In general, treatment should not be delayed beyond early third instar. The timing of application requires a subjective judgment weighing the extent of foliage development (typically 45-60 percent leaf expansion on red oak), larval stage of development (typically 30 percent first, 50 percent second, 20 percent third instar), the density of the population, and predicted level of pretreatment defoliation. Do not apply *Bt* to wet foliage. In addition to these biological factors, operational factors such as: (1) project objectives, (2) number of applications and (3) duration of larval hatch can have a bearing on the optimal timing of application(s).

Technology — Application equipment should be calibrated and characterized before every project. For calibration (determination of the flow rate) and characterization (the determination of spray drop size distribution and pattern), the actual tank mix should be used, especially, when using undiluted (neat) *Bt* as flow rates will vary from the nozzle manufacturer specifications, but in most cases water is used for calibration and characterization; therefore, drop sizes and flow rates need to be adjusted using correction factors (Reardon et al. 1991). Also, spray application equipment must be cleaned prior to this process as most *Bt* formulations, especially when applied undiluted, have a detergent action which will cause any residue in the spray system to flush into the nozzles. A Swath Kit has been developed to expedite calibration and characterization (Onken and Reardon 1990). The protocols for conducting characterization trials are available through the USDA Forest Service in Morgantown, West Virginia (contact the National Center of Forest Health Management at 304-285-1565), and the data from these trials should be forwarded to the same location for incorporation into a database. The optimal drop size and drop density of *Bt* on the target broadleaved foliage have not been determined yet. During application, however, a wide range of drop sizes 50-500 microns (µm) can usually be generated and deposited with the different types of nozzles and atomizers. Drop sizes between 75 and 200 µm volume median diameter (VMD) are usually used against the gypsy moth. Volume median diameter is the drop size with 50 percent of the emitted volume above and below this diameter. Also, data is insufficient to support the exclusive use of a particular atomizer or nozzle (e.g., flat fan, hollow cone, Micronair, Beecomist) over another. Therefore, a wide range of types and sizes are used for both rotary and fixed-wing aircraft (Reardon et al. 1991). In

general, rotary atomizers produce a narrower spectrum of drop sizes than other types of nozzles. In-line screens of 30 mesh or coarser should be used with undiluted *Bt* tank mixes. Fifty (50) mesh or coarser screens may be used with diluted tank mixes. Also, the use of slotted strainers instead of mesh screens are preferred for flat fan nozzles. Nozzle tips or atomizer screens should be checked and cleaned periodically to prevent excessive buildup or clogging, especially with the use of undiluted *Bt*. Also, undiluted *Bt* formulations can cause leaking problems with some types of aircraft pumps and the use of tungsten seals on the pumps tends to correct the leaking problems.

Dose and Volume —The current trend is towards increasing the dose and decreasing the total volume of *Bt* applied. Typically, doses of 40-90 BIU/ha (16 - 36 BIU/acre) are applied undiluted in volumes of 1.8 to 4.7 L/ha (24 to 64 oz/acre). The use of undiluted *Bt* provides adequate foliar coverage for greater production per aircraft load; no water carrier is required. *Bt* weighs approximately 8 to 10 lbs per U.S. gal. Since there exists only minimal replicated results statistically supporting the effectiveness of one dose and volume combination over others, there is a broad range of doses and diluted and undiluted volumes applied for control of gypsy moth.

Weather — There are generally accepted ranges in temperature (less than 80°F), relative humidity (greater than 50 percent), and wind speed (2 to 10 mph) recommended for aerial application of *Bt* to maximize deposit. These ranges are only broad guidelines as it is extremely difficult and expensive to accurately monitor these parameters at the aircraft spray height due to the tremendous amount of micro-variation. In general, weather conditions during the early morning hours are more preferable for maximizing deposit than those conditions prevalent at other hours during the day. (Anderson et al. 1992) compared the deposition of aerially applied *Bt* in an oak forest with predicted deposit using the Forest Service Cramer-Barry-Grim (FSCBG) canopy deposition and penetration model. The deposit concentration and spatial distribution of *Bt* were extremely variable among individual spray runs, primarily due to rapidly changing and somewhat unpredictable local atmospheric conditions. Nevertheless, the FSCBG model predicted the average *Bt* distribution accurately enough to demonstrate that it can be a reliable tool for estimating average spray deposition in broadleaved canopies.

Efficacy

Criteria — Eradication programs are designed to eliminate isolated populations of the gypsy moth. Suppression programs are designed to protect foliage or to reduce larval populations. A *Bt* suppression program may be considered "successful" in accomplishing one objective but not the other (i.e., foliage protection can be achieved without reducing the pest population to levels that would eliminate the need to respray the following season). Therefore, it is critical that program objectives are well defined with an adequate pre- and post-treatment monitoring program to accurately determine success.

Three parameters are usually used to measure field efficacy of *Bt* against gypsy moth: (1) population changes as measured by pre- and post-treatment egg mass densities, (2) comparison

of post-treatment larval and pupal densities between treated and untreated areas, and (3) estimation of post-treatment defoliation. Egg mass densities can be measured by one of several procedures (Kolodny-Hirsch 1986, Fleischer et al. 1992). Larval densities can be estimated by frass collections, or by tree band (e.g., burlap) counts; and defoliation can be estimated by ground observations or by aerial observation or photography (Figure 3).

Before 1970 — Metalnikov and Chorine (1929) first reported on the control of gypsy moth with *Bt*. This early report was verified approximately 30 years later (Cantwell et al. 1961). There were many annual field evaluations of *Bt* against the gypsy moth throughout the 1960's. Results of field studies at that time were highly variable and generally poor as formulations and application systems were in the initial stage of development (e.g., wettable powders tended to settle rapidly, booms and nozzles tended to clog) (Doane and Hitchcock 1964, Lewis and Connola 1966). In spite of frequent discouraging results, efforts to improve operational use of *Bt* intensified in part, because *Bt* was the only biological insecticide registered and commercially available for operational use against the gypsy moth.

1970's to 1994 — Shortly after the HD-1 strain became available in 1970, numerous yearly evaluations of combinations of doses, volumes, and number of applications of *Bt* began. Doses ranged from 2.5 to 40 BIU/ha (1-16 BIU/acre) and were applied in volumes that ranged from 4.7 to 37.6 L/ha (64oz/acre-4gal/acre). The treatments were applied once or twice at 4 to 10 days apart. Numerous experimental formulations (e.g., Thuricide HPC, Thuricide 16B, Dipel WP) and tank mix additives (e.g., molasses, antievaporants, stickers) were evaluated in the eastern United States (Secrest and McLane 1974; Dunbar and Kaya 1972; Kaya et al. 1974; Lewis et al. 1974; and Wollam and Yendol 1974). Collectively, these tests showed that *Bt* routinely provided acceptable foliage protection, but population reduction to densities of less than 620 egg masses (EM)/ha (250 EM/acre) was inconsistent or rarely achieved. These inconsistent results were thought to be due, in part, to staggered egg eclosion and development of the gypsy moth, limited formulation and application technology, and the moderate susceptibility of larvae to *Bt* (Dubois 1981, Dubois and Lewis 1981).

During the late 1970's, the recommended protocols for aerial application of *Bt* were two applications, each at a dose of 20 BIU/ha (8 BIU/acre) and applied at a volumetric rate of 9.3 to 18.7 L/ha (1-2 gal/acre). The first application was made when the majority of the larvae were in the first instar, and the second application was made 7-10 days later when eclosion was complete, the larvae matured to second and third instars, and oak foliage was 50-80 percent expanded. These early efforts provided consistent foliage protection but again failed to consistently reduce populations. Also, the double applications further increased the cost of treatment and complicated the logistics for large-scale spray operations.

Harper (1974) suggested that one application of *Bt* was adequate for foliage protection but two applications should be used in certain situations, such as when:

1. Population density is abnormally high.
2. The hatching period is usually prolonged.

3. Cold weather follows application (where larvae do not feed but physical degradation of the product takes place).
4. Foliage development is delayed in relation to larval growth, resulting in little foliage surface area and low spray retention.
5. Rain washes away the first application (i.e., rain within 6-8 hours after treatment).
6. Post-treatment immigration of larvae into the sprayed area is significant.

Efforts that were initiated in the 1970's intensified in the 1980's, and focused primarily on formulation development and application technology to improve the efficacy and consistency of population reduction with a single application. Improved higher potency oil-based and water-based formulations of *Bt* with extended residual activity became available.

In 1981, Dubois isolated another *Bt* var. *kurstaki* strain from diseased spruce budworms and named it NRD-12 (Dubois 1985). Laboratory bioassays indicated that it was more potent and killed gypsy moth faster than did the HD-1 strain. The NRD-12 isolate was used in several commercial products introduced in the mid 1980's, including SAN 415 SC 32LV and Javelin by Sandoz Inc. In 1986, Dubois et al. (1988) evaluated the SAN 415 SC 32LV formulation against gypsy moth populations in Maryland. Both one and two applications at 30 BIU/ha (12 BIU/acre) were equally effective in providing significant foliage protection and population reduction. In 1989, the San 415 SC 32LV formulation was aerially applied against low-density (less than 275 EM/ha) gypsy moth populations using two neat applications, each at the dose of 49.4 BIU in 6.0 L/ha. Results of this study demonstrated the potential of SAN 415 to suppress the growth of low-density gypsy moth populations; control plots showed an overall 55-fold increase in EM density compared with only a 3.1-fold increase in EM densities inthe SAN 415 plots (Podewaite et al. 1993). Presently, only the Sandoz product SAN 415 SC 32 LV is registered for use against forest pests.

In 1983, field studies and operational spray programs evaluated many formulations of *Bt* (e.g., Dipel 4L, 6L and 8L; Thuricide 32LV, 48LV) applied at several doses including 30, 40, 50 BIU/ha (12, 16 or 20 BIU/acre) and at volumetric application rates of 1.8, 2.3, 3.5,4.7, or 9.4 L/ha (24, 32, 48, 64 or 128 oz/acre). Results indicated that, when properly applied, 30 BIU/ha (12 BIU/acre) or higher at rates of 7.0 or 9.4 L/ha (96 or 128 oz/acre) provided foliage protection comparable to that obtained with chemical insecticides. In addition, double applications of *Bt* were no more effective than a well-timed single application in protecting foliage.

From these early observations, Lewis (1984) remarked that increasing the dose (BIU/ha) above a minium threshold level did not necessarily improve efficacy or consistency, that larval development in treated plots was slower than in the control plots, and that larger numbers of *Cotesia melanoscelus* (Ratzeburg) (Hymenoptera: Braconidae) cocoons were recovered in the *Bt* treated areas than the untreated areas.

Concurrently, during the late 1970's and early 1980's, results of research and operational efforts that used *Bt* to control spruce budworms in both the United States and Canada began to

influence the use of *Bt* for control of gypsy moth (Cunningham and van Frankenhuyzen 1991, and van Frankenhuyzen 1990). In 1978 the recommended dosage of *Bt* for spruce budworm control was 20 BIU/ha (8 BIU/acre) in 4.7 L/ha (64 oz/acre). By 1990, the dosage had increased to 30 BIU/ha (12 BIU/acre) and volumes were reduced to as low as 1.6 L/ha (20 oz/acre). One 50 um droplet of a 12.7 BIU/L product per balsam fir needle would provide effective spruce budworm control with an efficacious dose of toxin in each droplet. Small droplets of 15 to 55µm VMD seemed optimal for impingement on coniferous foliage and on silk strands that served as feeding shelters for the spruce budworm as this might be an important dose transfer mechanism. Current beliefs are, however, that these small drops do not have sufficient volume to contain an effective dose. Effective use of small droplets requires efficient atomization of the spray formulation and the availability of nonvolatile formulations (van Frankenhuyzen 1990).

Between 1980 and 1984, only 2 to 4 percent of the total area sprayed for spruce budworm was treated with *Bt*; it increased to 63 percent in 1990.

Between 1985 and 1990, 204,000 ha were treated with *Bt* in Ontario to control gypsy moth. This increased use was attributed to use of higher doses and lower volumes, better formulations, and improvements in application technology (Cunningham and van Frankenhuyzen 1991).

With the availability of high potency formulations (20 BIU/L = 76 BIU/gal) of *Bt*, there were numerous studies in the Northeast evaluating the effect of application parameters on the efficacy of *Bt*. In 1988, using water as a diluent, Mierzejewski et al. (1993) evaluated one dose of *Bt* applied at three volumetric rates (undiluted at 3.5L/ha, and diluted at 9.4 L/ha, and 18.7 L/ha) on replicated 20-ha plots in West Virginia. All three treatments provided comparable foliage protection, population reduction, and deposit efficiency. These observations, as well as those from other sources, indicated that *Bt* could be applied in lower volumes and provide acceptable efficacy at reduced cost.

Over the next 4 years, from 1989 through 1992, a series of studies was conducted to increase the efficacy of *Bt* through improvement of application technology. In 1989, Dubois et al. (1993) compared the efficacy of three doses of *Bt* and the influence of volume on deposit efficiency and efficacy. All treatments significantly reduced the population and protected the foliage. At 90 BIU/7.0 L/ha, the pest population was consistently reduced (five replicates out of five) to less than 124 EM/ha (<50 EM/acre). Though the mean population reduction (percent control) at doses of 30 BIU applied at 2.3 L (undiluted) or 7.0 L/ha and of 60 BIU applied at 4.6 L/ha (undiluted) did not differ significantly than 90 BIU/7.0 L/ha, they were not as consistent; i.e., only one or two replicates out of five were reduced to less than 124 EM/ha (<50 EM/acre). When the low dose (30 BIU) was compared at low undiluted (2.3 L/ha) or at high (7.0 L/ha) volumes using an inert carrier as the diluent to maintain the same viscosity and specific gravity, both the mean number of drops/cm^2 and volume deposited (nanoliters/cm^2) were significantly greater at the higher volume application rate. Also the percent of leaves (of 800 leaves sampled) with five or more drops per cm^2 leaf surface was significantly greater at higher volume application (56.5 percent) than at low volume (7.9 percent). Finally at all drop density groups (<1, <5, <10 <20

and>20 drops/cm^2), most of the detectable drops landing on foliage were between 75 and 150 µm VMD.

These studies were followed with evaluations of the effect of nozzle types on the efficacy of *Bt* (Dubois et al. In press, Smitley and Davis 1993). In the Dubois et al. (In press) study, three nozzles were compared with each other: the AU5000, Flat Fan with 8004 tips, and Twin Jet 8004. Use of all three nozzles significantly reduced the larval population and prevented defoliation when compared with the untreated controls. In the Smitley and Davis (1993) study, the differences in susceptibility of *Populus* and *Quercus* species to defoliation and duration of outbreaks in these species by gypsy moth complicated their evaluation of treatment effects.

In 1991 and 1992, studies were conducted to expand the *Bt* spray window by treating third and fourth instar gypsy moth larvae. Studies (Dubois et al. In press) conducted over the 2-year period indicated that Foray 48B applied undiluted at 90 BIU/ha, significantly reduced the larval populations and prevented further defoliation, but Thuricide 48LV or 64LV applied at 99 BIU/ha did not. Thuricide 64LV applied at 99 BIU/ha at two drop sizes, 110 and 163 µm VMD, both significantly reduced the egg mass numbers but the residual population remained unacceptably high. Foliage protection was generally acceptable in all treatments.

Minimal effort has been allocated to conducting research and methods improvement activities concerning the ground application of *Bt* (Yendol et al. 1973). Dubois (1971) evaluated two commercial formulations of *Bt* using a mist blower that provided excellent foliage protection and population reduction. Dubois and McLane (1991) compared the efficacy of *Bt* when applied with mist blower and hydraulic sprayer. The volume used with the mist blower ranged between 94 and 281 L/ha (10-30 gals/acre), and the volume used with the hydraulic sprayer, ranged from 468 to 935 L/ha (50-100 gal/acre). At both doses tested, 30 and 60 BIU/ha (12-24 BIU/acre), treatment with the mist blower but not the hydraulic sprayer significantly and consistently reduced the larval population and minimized defoliation.

In general, the aerial application of *Bt* provides good foliage protection, but population reduction is highly variable (Dubois et al. 1993, Smitley and Davis 1993). Also, there is insufficient data to recommend the aerial application of *Bt* over another insecticide for various gypsy moth population densities in an effort to maximize efficacy. Current research to reduce these constraints is focused on development of higher potency *Bt* products with greater foliar persistence and on improving product by selection and commercialization of more effective strains. The enhancement of natural strains by genetic manipulation (engineering or other selection techniques) offers exciting prospects for improvements in the longer term (van Frankenhuyzen 1990).

Deposition — There has been an increased effort to quantify deposition of *Bt* on the target foliage and to use these results as a general predictor of treatment efficacy. In the laboratory, Yendol et al. (1975) showed that when given a choice, gypsy moth larvae consumed more untreated leaf disks or those sprayed with the lowest *Bt* concentrations than those receiving the

highest concentrations. Also, larvae discriminated between the highest and lowest *Bt* concentrations even with the addition of molasses as a feeding stimulant. Bryant and Yendol (1988) showed that a given dose of *Bt* per unit oak leaf surface area (cm^2) was more effective when applied at a higher density of small drops (50 to 150 µm) than at a lower density of larger drops (>150 µm). Also, Radcliffe and Yendol (1993) documented that for third instar gypsy moth larvae the LD_{50} was 2.7 (range 1.9 - 3.4) IU/larva and the LD_{95} was 21.1 (13.6 - 48.5) IU/larva. After larvae consumed a lethal dose of *Bt*, the mean time to death ranged from 37.7 to 45.2 hours.

Yendol et al. (1990) showed that the distribution of *Bt* deposit within a broadleaved forest canopy following aerial application was highly variable; however, deposit differences between upper and lower canopy levels or directionally within canopy level, were not significant. Spray is not deposited uniformly between leaves. Instead, deposition tends to be log normal, where many leaves contain less than the average dose, balanced by relatively few highly dosed leaves.

A qualitative estimate of the spray deposit can be made by determining drop density on the target foliage or on exposed artificial collection surfaces, such as Kromekote cards or water or oil sensitive cards. For such estimates with Kromekote cards, a dye is usually incorporated into the tank mix. The dye selected for use needs to be compatible with the *Bt* formulation and not affect feeding by the target pest. Techniques to quantify the actual insecticidal activity deposited are labor intensive and still in the experimental stage. The specific techniques used would depend on objectives, budget, and other factors. For an operational spray program, we recommend a combination of techniques: (1) incorporating dye into the tank mix and measuring volume and drop density on target leaf surfaces, (2) placing gridded filters (Millipore Corp; RI) to collect drops containing viable *Bt* spores, which are counted as viable *Bt* colonies after incubation on Trypticase Soy Agar (used to determine viable deposit as underestimates the actual spray dosage), and (3) collection and bioassay of foliage from sprayed trees to quantify insecticidal activity.

Persistence — Loss of residual toxicity of *Bt* can result from degradation by sunlight, leaf temperatures, drying, being washed off by rain, microbial degradation, and leaf chemistry (Kushner and Harvey 1962, Pinnock et al. 1975, Leong et al. 1980, van Frankenhuyzen and Nystrom 1989, Beckwith and Stelzer 1987). Solar radiation appears to be the key factor affecting survival of *Bt* spores and crystals deposited on foliage (Morris 1983, Pozsgay et al. 1987). The half-life for *Bt* spores was estimated at 3.8 hours when exposed to an uninterrupted ultraviolet source representative of the ultraviolet spectrum in natural sunlight (Ignoffo et al. 1977). Some tree leaves contain substances that inhibit *Bt* toxicity when mixed together in the insect midgut. In a series of *Bt* bioassays, (Sundaram et al. 1992) estimated the half-life of *Bt* insecticidal activity in the field at 12-22 hours. Other estimates of the half life of *Bt* insecticidal activity have been calculated at 24-32 hours (Dubois 1993a). In spite of the short half-life of *Bt*, a deposition of 75 IU/cm^2 from a 90 BIU/ha application will give, on the average, insecticidal activity of at least an LD_{50} for 4 to 6 days.

Safety

Many safety tests have been performed with *Bt* (Otvos and Vanderveen 1993). It has been inhaled, injected, fed to, or scratched onto many different animals including rats, mice, swine, rabbits, guinea pigs, dogs, and chickens. None showed any abnormal reaction attributed to the *Bt* in terms of external symptoms or internal pathologies (Harper 1974, USDA Forest Service 1989, Bobrschmidt et al. 1989). An exhaustive study on the effect of *Bt* sprayed on humans during multiple applications of *Bt* over urban populations to control the Asian strain of the gypsy moth in British Columbia, showed no adverse effect of *Bt* on humans (Noble et al. 1992).

Microbial Contaminants

The presence of microbial contaminants, often referred to as "bioburdens", in *Bt* liquid flowable concentrates has always been a concern to *Bt* manufacturers, particularly for those bacteria and yeasts that could be pathogenic to nontarget plants and animals, especially humans. Since 1989, implementation of rigid quality control measures and intense scrutiny of commercial products have failed to detect significant presence of bioburdens in *Bt* flowable concentrates. This observed level of product quality was not always the case. Concerns for the presence of microbial contaminants surfaced in 1987 from reports that some commercial formulations of *Bt* examined between 1985 and 1987 contained a significant number of bacterial or yeast microbes or both. None of these microbes presented a threat to human health; however, they could potentially become threats to users, insects other than the target pest, or other components of the environment. The stability and potency of the *Bt* formulations may also be adversely affected by these organisms growing in the formulation concentrates.

For 3 to 4 years, random samples of *Bt* formulation lots purchased by personnel with various State Departments of Agriculture and Forestry — particularly Pennsylvania, Michigan, New Jersey — and the Canadian government were sampled for the presence of these contaminants. The contaminants found included other *Bacillus* sp., *Micrococcus* sp., *Sarcina* sp., *Staphlyococcus* sp., *Streptococcus* sp. and *Candida* sp. None of these were considered pathogenic or threatening, nor were they present in high density. Nonetheless, their presence was symptomatic of poor quality control by the manufacturers and represented an avenue through which other possibly pathogenic or opportunistic microbes could be transmitted to the environment or to users and others exposed to the sprays. Immediate remedial action was taken by the manufacturers, who since 1988, have guaranteed that their products are free of detectable levels of bioburdens when purchased. Continued sampling through 1990 supported this declaration, and no significant concentrations in levels of bacterial or yeast contaminants have been found in any *Bt* products of the major manufacturers.

Though a potentially unpleasant situation was averted, this experience should continue to remind users to use care when handling and using *Bt* formulations even though these formulations are produced under stringent conditions and contain bacteriostats and other agents to prevent the

possibility of buildup of contamination. Careful handling, especially important when seals on containers are broken and the contents are exposed to air contaminants for any length of time (though these would not be expected to establish themselves in the concentrates), using previous years' production, and especially when water of unknown potable quality is used as a diluent in a tank mix.

Effects on Nontarget Organisms

There have been numerous, short-term (1-3 years) field studies conducted to determine potential effects of *Bt* on nontarget organisms. Unfortunately, most of these studies were conducted as a minor component of an operational program and in general, data suffer from lack of adequate funding, replicated areas, and sampling techniques. Also, only one application of *Bt* was applied for most of these studies and there is minimal nontarget data for double applications applied in one year or sequential yearly applications of *Bt*. Since *Bt* var. *kurstaki* is used to control lepidopteran pest species, it is assumed that lepidopteran species other than gypsy moth would also be affected.

In Oregon, Miller (1990) observed reductions in both species richness and abundance of nontarget Lepidoptera after multiple (three) applications of *Bt*. In another study, biomass of lepidopteran larvae was reduced by application of *Bt*, and birds in the treated areas made significantly fewer nesting attempts (Rodenhouse and Holmes 1992). Johnson et al. (In press) observed that *Bt* was toxic to first and second stage tiger swallowtail, *Papilio glaucus* (L.), larvae for up to 30 days.

Food of the Virginia Big-eared Bat — A 3-year study (pre-treatment, 1990; treatment, 1991; post- treatment, 1992) was initiated in 1990 in West Virginia to determine the potential effects of *Bt* on food of the endangered Virginia big-eared bat (a mammal that feeds almost exclusively on moths). *Bt* was applied undiluted at the rate of 7.0 L/ha (96 oz/acre) and dose of 90 BIU/ha (36BIU/acre) in one application. Deposition and persistance of *Bt* residues were determined by insect bioassay (Sundaram et al. 1992). In general, *Bt* effects on lepidopteran larvae were most evident in 1991, the year of application, as species richness and abundance were reduced. Nevertheless, fluctuations in populations of lepidopteran larvae of equal impact were recorded as a result of climatic conditions (hot, dry weather in 1991 and cool, wet weather in 1992) (Sample et al. In press). Because of the short residual activity of *Bt*, the greatest impacts were among those species collected within 3 weeks of application. No moth genera on which the bats feed were significantly less abundant after *Bt* application.

Lepidoptera in Broadleaved Forests — A 3-year study (pre-treatment, 1991; treatment, 1992; post-treatment, 1993) was initiated in 1991 in Virginia to determine the potential effects of *Bt* on native Lepidoptera. A single application of *Bt* [Foray 48B, 90 BIU/ha (36 BIU/acre)] was evaluated on 10, 20-ha plots in Rockbridge County, Virginia. Five plots were treated and five plots were left untreated. Larvae of native Lepidoptera were sampled in treated and untreated plots using two methods: (1) taking foliage samples from the canopy (scarlet oaks), mid-canopy (scarlet oaks), and understory (blueberry); and (2) placing burlap bands at eye level on the trunks of scarlet or chestnut oak (Wagner et al. In press.)

Over 10,000 larvae (about 500 macro-lepidopteran larvae and over 9600 micro-lepidopteran larvae) were collected on foliage samples in 1992. For both macro- and micro-larvae, there appeared to be a treatment effect for samples collected after *Bt* application.

Over 700 macro-larvae were collected under burlap bands in 1992; about 1200 macro-larvae were taken under burlap banding in 1993. On nearly every sampling date in both 1992 and 1993, there appeared to be significantly fewer macro-larvae under burlap bands on oaks in treated plots than in untreated plots.

Interaction with Natural Enemies

Larval mortality caused by nucleopolyhedrosis virus (NPV) was lower among larvae collected from plots treated with *Bt* than in adjacent untreated plots (Webb et al. 1989, Woods et al. 1988). The *Bt* treatment reduced the density of early-instar NPV-killed cadavers and the amount of viral inoculum released to the residual larval population.

Increased rates of parasitism by *C. melanoscelus* are frequently observed in areas treated with *Bt* (Andreadis et al. 1983, Weseloh et al. 1983). This enhancement of parasitism by *C. melanoscelus* and negative influence on parasitism by *Compsilura concinnata* and *Blepharipa pratensis* (Diptera:Tachinidae), was also documented for reduced application rates of *Bt* (Ticehurst et al. 1982). Reduced rates of parasitism by *Brachymeria intermedia* (Hymenoptera:Chalcididae) in *Bt*-treated plots, were probably due to the photopositive behavior of adult females (Andreadis et al. 1983 and Reardon et al. 1979).

Both laboratory studies using sublethal doses of *Bt* and field measurements of larval development rates in blocks aerially sprayed with *Bt*, have confirmed that the mechanism causing the synergism between *C. melanoscelus* and *Bt* is delayed larval development resulting from temporary gut paralysis after ingestion of sublethal doses of *Bt*. The temporal delay of approximately 1 week in the development of larvae persisted throughout the larval feeding period and by the 6th week after *Bt* application, larvae in untreated areas began to pupate, while in *Bt* treated areas most larvae were still in the fourth and fifth instars. This synergistic effect suggests that inundative releases of *C. melanoscelus* together with *Bt* might be more effective than separate application of *Bt* and parasite against the gypsy moth (Wollam and Yendol 1976, Ahmad et al. 1978, Ticehurst et al. 1982, Wallner et al. 1983, Andreadis et al. 1983).

New Developments

Insecticidal Crystal Proteins

Hofte and Whiteley (1989) first classified the insecticidal crystal proteins (ICP's) of *Bt* and named them according to the genes that encode them. The cryI(A to E) groups with their subgroups, e.g., cryIA(a,b or c), cryIIB and cryIIA (which is also toxic to dipteran larvae) are toxic to lepidopteran larvae. The cryIIIA is toxic to Coleoptera and the cryIV(A to D) group is toxic to dipteran larvae.

The commercial formulations produced with the HD-1 strain generally contain one or up to three of the cryIA ICPs. Recent studies of these purified ICPs against the gypsy moth showed that cryIA(a) and cryIA(b) are significantly more toxic than cryIA(c). This is not necessarily the case with all lepidopteran larvae. Results also indicate that at high concentrations, cryIA(a) may become dose-limiting where maximum mortality does not exceed 85 percent. The addition of a very small amount of spores to a low concentration of the ICPs, however, significantly increases mortality to 100 percent as a result of lethal septicemia. Spores alone have no effect on gypsy moth larvae. This interaction between bacteria and the ICPs does not appear to be specific to *Bt* spores. Many but not all bacteria that are part of the microflora of the forest environment also show significant synergism with the cryIA(a) and cryIA(c) ICPs (Dubois and Dean 1993). These observations suggest that once the midgut is perforated, these insects become very susceptible to nonspecific infections by bacterial opportunists and that the forest microflora can act synergistically with *Bt*.

Resistance

Development of resistance to chemical insecticides has been observed since 1908. Development of resistance to *Bt,* however,was first reported in a laboratory study where intensive selection pressure was applied against the Indianmeal moth, *Plodia interpunctella,* (McGaughey 1985). Since then several other insect species were similarly selected for resistance under intensive laboratory selective pressures. Lepidoptera selected include the tobacco budworm, *Heliothis virescens*; almond moth, *Cadra cautella*; and sunflower moth, *Homoeocosoma electellum* (McGaughey and Whalon 1992). To date, only one insect species, the diamondback moth, was reported to have developed resistance to *Bt* when used in operational spray programs. Against diamondback moth, such spray programs required multiple applications (five to eight) per generation with as many as eight to ten generations per year (Tabashnik et al. 1990). The following have been suggested as explanations for resistance development to *Bt* toxins in formerly susceptible hosts: Behavior and midgut physiological changes (McGaughey and Whalon 1992), as well as molecular changes in the midgut membrane receptors (to which the toxins no longer bind) (Van Rie et al. 1990, Gould et al. 1992, Ferre et al. 1991).

Tabashnik (1989) recommended multiple pesticide application tactics as a possible method to manage resistance. Rossiter et al. (1990) investigated the feasibility of development of resistance to *Bt* in gypsy moth and found that significant variation in susceptibility between egg masses (families within a population) suggested a potential for resistance development through natural selection. Most of the variation (i.e., 42 percent), however, was the result of ovipositioning sequence (egg laying), where the eggs laid first were less susceptible than the eggs laid last and only 16 percent was attributed to family differences. Though the potential for selecting for resistance does exist, this genetic factor is very much mitigated by maternal provision and environmental factors.

Dubois (1993b) reviewed the feasibility of development of resistance to *Bt* in forest protection and found that there were some significant differences between gypsy moth and the species that had developed resistance:

1. The gypsy moth has only one generation per year.
2. *Bt* is usually applied 1 to 2 times per generation (there is minimal selective pressure).
3. Populations are not isolated (i.e., gene flow is not restricted).
4. Susceptibility is complex (involves more than one toxin and septicemia from the spore).
5. Population behavior, dispersion, and intermixing assures a gene pool for heterozygotes and a wild population refugium.
6. *Bt* has a short field persistence and natural viral epizooties reduce selected resistance.

Based on the evidence available to date, the gypsy moth is unlikely to develop significant resistance to *Bt* in the foreseeable future when used in operational spray programs.

Technology

The demand for development of environmentally friendly biopesticides, along with the technical developments in genetic engineering and molecular biology during the 1980's, have provided opportunities for development of biopesticides in a variety of ways. In addition to the four manufacturers already mentioned (Abbott, Ecogen, Novo Nordisk and Sandoz), over 40 other companies worldwide are involved in the development and manufacture of biopesticides (Goettel 1991, 1993). Collectively these manufacturers produce over 60 products targeting over 90 pests that attack over 80 different crops. Eighteen manufacturers are involved in developing natural strains of *Bt*, genetically manipulated strains, and transfer of toxin-coding genes into other bacteria (cloning) or plant species (transgenic plants) (Feitelson et al. 1992). The major producers of *Bt* products used in forestry (i.e., Abbott, Novo Nordisk and Sandoz) still use *Bt* strains developed from natural isolates.

Product improvement through genetic manipulation is already being attempted. Condor OF produced by Ecogen is a genetically altered *Bt* strain developed through transconjugation techniques (a natural transfer of genetic material from a donor *Bt* bacteria to a recipient *Bt* by cell mating). In laboratory analysis, Ecogen produced a strain that was more potent to the gypsy moth than are currently used strains. For a number of reasons, however, that expected increased efficacy was not realized when a formulation of the strain was used operational under field conditions.

A second example of utilizing new technology to develop new *Bt* toxin products is the development of MVP (*Btk*) and M-Trak (from *Bt* var. *tenebrionis*) by Mycogen Corp. These products are made from the genes of the *Bt* strains which are cloned into a *Pseudomonas fluorescens* cell, which is chemically killed, and whose cell wall then acts as a capsule stabilizing the toxin from degradating factors in the environment. Monsanto Corp. and others have cloned *Bt* toxins into root-colonizing bacteria to protect plants from root-feeding pests, and successfully inserted *Bt* toxin genes into plants. Because of the distinct possibility of development of resistance to *Bt* toxins with these strategies, the companies are also developing other strategies to delay the development of resistance.

These are only a few examples of the diverse development of *Bt* and the promise it holds in the development of natural strains and new products to protect natural resources in an environmentally acceptable manner.

Conclusions

The development of environmentally benign tactics for use in Integrated Pest Management (IPM) programs to manage agricultural and forest pests will continue as a major area of research. *B. thuringiensis* will continue to be an important tactic in IPM programs, although genetically manipulated *Bt* products will be developed for specific pest species. For *Bt* and for biologicals in general, there is an urgent need to improve residual activity of deposit on the target, develop an operational assessment method for deposit, and to develop specific aerial application technology.

Summary

Bacillus thuringiensis was first isolated in the early 1900's. In the United States, commercial production of *Bt* (as *Bt* var. *thuringiensis*) was initiated through the efforts of E. A. Steinhaus. In 1970, Dulmage isolated the HD-1 strain of *Bt* var. *kurstaki* and it became commercially available in the early 1970's. This strain is often referred to by its acronym "*Btk*" and is used today for production of most *Bt* formulations used to control defoliating forest Lepidoptera in North America.

Commercial formulations of *Bt* contain both the spore (or endospore) and crystal (or parasporal body). The crystal is a protein matrix of large molecules of inactive protoxins that are not toxic to insects until solubilized in the insect gut and released as smaller protein delta-endotoxins. These proteins, also known as the insecticidal crystal proteins (ICPs), bind to and force through specific receptor sites on the midgut membrane forming an ion-selective channel. The selective permeability of the membrane is disrupted, causing the cell to absorb water, swell and burst. Gut paralysis occurs, and the larva stops feeding and dies.

The potency of *Bt* preparations is determined by parallel bioassays with the standard (HD-1-S-1980) on artificial diet with 4-day-old cabbage loopers. Since insecticidal activity against a diversity of insect species varies greatly, this method often results in a misrepresentation of the actual efficacy against species other than the cabbage looper. For gypsy moth, parallel bioassays with the standard and test preparations are conducted against 1-day-old second-instar larvae.

The effective use of *Bt* to control gypsy moth involves the interaction of numerous factors (e.g., formulation, deposit characteristics, application timing and technology, and weather). To date, for gypsy moth control in broadleaved forests, the most efficacious deposit (VMD, drop density), dose and volumetric rate of application have not been identified. Therefore, a variety of aircraft types, nozzles and atomizers are used, and a broad spectrum of formulations, dose and volumetric rates are aerially applied. The current trend is to apply *Bt* at higher doses (60-90 BIU/ha) and at lower undiluted volumetric rates (3-5 L/ha) for one application. Efforts to improve efficacy might be

directed at decreasing loss of residual activity, and selection and commercialization of more effective strains.

The safety of *Bt* for vertebrates is well documented in many laboratory tests and specifically on humans during multiple applications of *Bt* over urban populations in British Columbia. Reductions in species richness and abundance of some nontarget lepidopteran larvae were detected for *Bt*.

Increased rates of parasitism by *C. melanoscelus* are frequently observed in areas treated with *Bt*. The mechanism causing this synergism is delayed gypsy moth larval development resulting from temporary gut paralysis after ingestion of sublethal doses of *Bt*.

The development of resistance to *Bt* was first reported in a laboratory study where intensive selection pressure was applied against the Indianmeal moth. To date, only the diamondback moth has developed resistance to *Bt* when used in operational spray programs. Based on available evidence, the gypsy moth is unlikely to develop significant resistance to *Bt* in the foreseeable future when used in operational spray programs.

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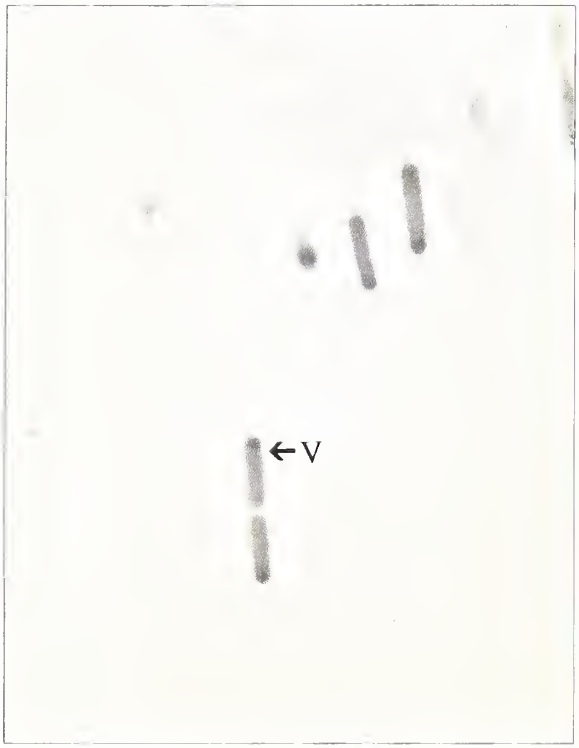
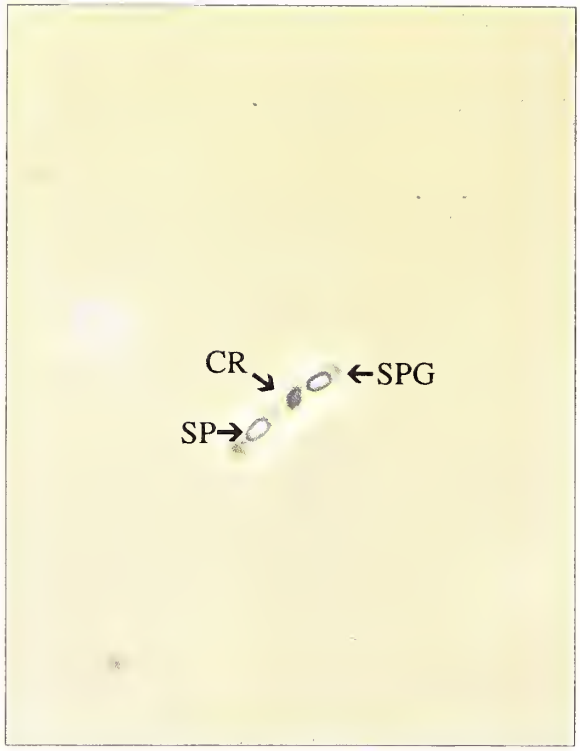
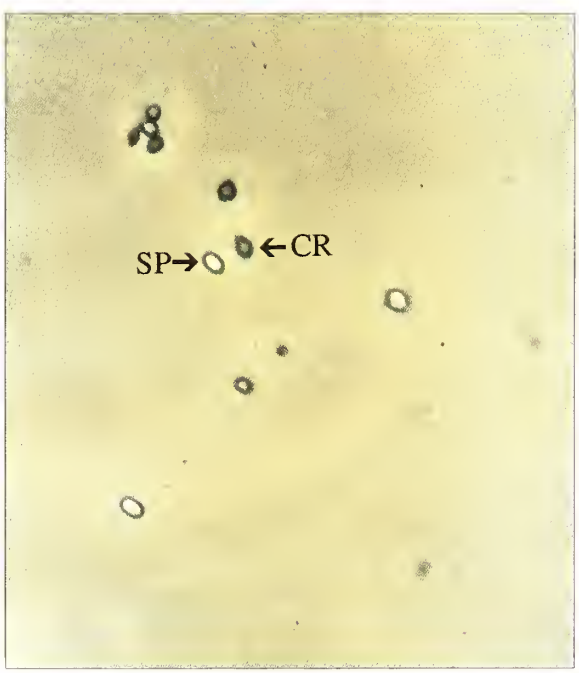


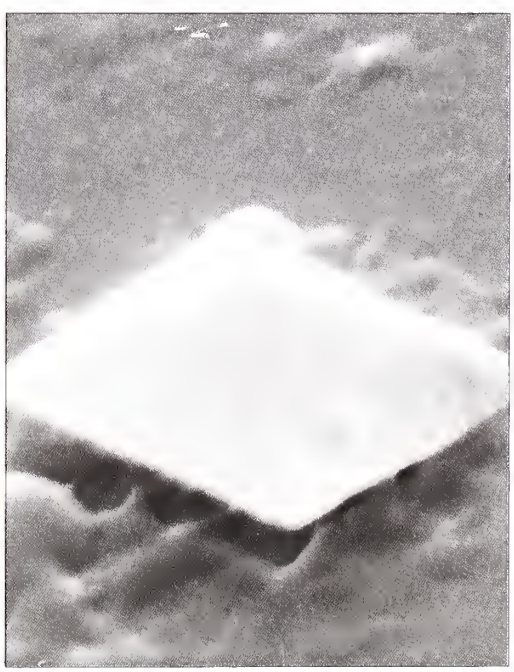
(**A**) vegetative growth stage;
v = vegetative cells



(**B**) sporulating stage; SPG = sporangium,
SP = spore, CR = crystal (delta-endotoxin)



(**C**) released spores and crystals



(**D**) scanning electromicrograph of crystal
(× 16,000)

Figure 1. Development stages of *Bacillus thuringiensis*:

A

B

Figure 2. (A) Aerial application of *Bacillus thuringiensis* to control gypsy moth; (B) *Bacillus thuringiensis* deposit and killed gypsy moth larvae on foliage.

Figure 3. Aerial view of area treated with *Bacillus thuringiensis* and untreated area surrounding treatment.

Pesticide Precautionary Statement

This publication reports the aerial application of insecticides. It does not contain recommendations for insecticide use, nor does it imply that the uses discussed here have been registered. All uses of insecticides must be registered by appropriate State and/or Federal agencies before they can be recommended.

Caution: Insecticides may be injurious to humans, domestic animals, desirable plants, and fish or other wildlife if they are not handled or applied properly. Use all insecticides selectively and carefully. Follow recommended practices for the disposal of surplus insecticides and insecticide containers.

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